Invariant NKT Cells Suppress CD8$^+$ T-Cell–Mediated Allergic Contact Dermatitis Independently of Regulatory CD4$^+$ T Cells

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Invariant natural killer T (iNKT) cells expressing a CD1d-restricted invariant αβTCR have key regulatory roles in autoimmunity, pathogen immunity, and tumor surveillance, but their function in the control of allergic skin diseases remains poorly documented. Using a model of contact hypersensitivity (CHS) to the hapten DNFB, we show here that iNKT cell deficiency results in enhanced skin inflammation due to augmented hapten-specific IFN-γ-producing CD8$^+$ effectors in skin draining lymph nodes (dLNs) and their massive recruitment into the allergen-exposed skin. Adoptive transfer and antibody depletion experiments as well as *in vitro* studies revealed that iNKT cells (1) reduce the severity of CHS, even in presensitized mice, (2) require hapten presentation by CD1d$^+$ dendritic cells (DCs) to dampen skin inflammation, and (3) produce IL-4 and IL-13 after CD1d-dependent *in vitro* stimulation by hapten-loaded DCs only in the presence of IFN-γ released from activated CD8$^+$ effector T cells. In corollary, mice double deficient in IL-4 and IL-13 exhibit an exacerbated CHS. Finally, iNKT-suppressive function is independent of Foxp3$^+$ regulatory T cells (Tregs). These data highlight that, besides Foxp3$^+$ Tregs, iNKT cells are potent downregulators of CD8$^+$ T cell–mediated CHS, and underscore that both cell types are important for the regulation of allergic skin inflammation.

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INTRODUCTION

Invariant natural killer T (iNKT) cells are a distinct lineage of T cells that coexpress invariant TCRα chains (Vα14Jα18 in mice and Vα24Jα18 in humans) and natural killer receptors, and that are reactive to lipids/glycolipids presented by the major histocompatibility complex class I–like molecule, CD1d. Another class of lipid/CD1d-restricted T cells, referred to as variant NKT or type-II NKT cells, express variable (noninvariant) TCRs (Godfrey and Berzins, 2007). Mouse invariant NKT (iNKT) cells constitute up to 30–40% of TCRαβ$^+$ T cells in the liver. They recognize glycolipids presented by CD1d expressed by various types of dendritic cells (DCs), including dermal DCs (Gerlini *et al.*, 2001) and epidermal Langerhans cells (Fukunaga *et al.*, 2010). CD1d ligands activating iNKT cells include synthetic α-galactosylceramide (α-GalCer), microbial lipids, and endogenous ligands (Bendelac *et al.*, 2007). α-GalCer stimulation of iNKT cells induces the release of IFN-γ and IL-4, which enhance the function of DCs and T cells, linking innate and adaptive immunity (Bendelac *et al.*, 2007). Although iNKT cells can stimulate T-cell immunity to pathogens and tumors (Bendelac *et al.*, 2007), they can act as regulatory cells in autoimmunity (Novak and Lehuen, 2011), peripheral, and mucosal T-cell tolerance (Sonoda *et al.*, 1999) and tumor surveillance (Taniguchi *et al.*, 2010). Such dual functionality of iNKT cells has not been so far documented in allergy. Indeed, iNKT cells were reported to promote airway hyperreactivity by licensing Th2 (T helper type)-type cell entry into the lung (Akbari *et al.*, 2003; Lisbonne *et al.*, 2003), to mediate contact hypersensitivity (CHS) to picryl chloride (Campos *et al.*, 2003), and to infiltrate the inflamed skin of patients with allergic contact dermatitis (Gober *et al.*, 2008). However, recent evidence that iNKT cells can dampen T-cell responses to skin antigens (Fukunaga *et al.*, 2010; Mattarollo *et al.*, 2011) suggests that they might also inhibit CHS responses reproducing allergic contact dermatitis.

CHS is induced by epicutaneous challenge of mice, topically presensitized to strong haptens such as DNFB, which become immunogenic after covalent binding to self-proteins. We and others have documented that CHS to DNFB is mediated by allergen-specific class I–restricted CD8$^+$ T cells (Bour *et al.*, 1995; Xu *et al.*, 1996), which are primed in skin draining lymph nodes (dLNs) and differentiate into

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Abbreviations: BM-DC, bone marrow–derived dendritic cell; CHS, contact hypersensitivity; DC, dendritic cell; dLN, draining lymph node; DNBS, 2,4-dinitrobenzene-sulfonic acid; α-GalCer, α-galactosylceramide; iNKT, invariant natural killer T cell; LN, lymph node; Treg, regulatory T cell; WT, wild type

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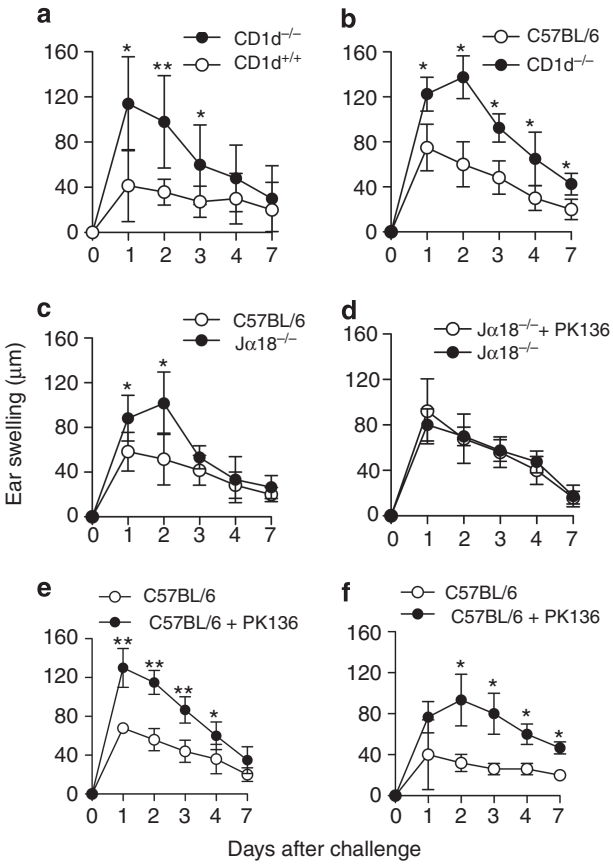


Figure 1. Contact hypersensitivity (CHS) to DNFB is downregulated by invariant natural killer T (iNKT) cells. CHS to DNFB was tested in various settings. (**a**, **b**) CHS response of CD1d$^{-/-}$ (black circle) compared with CD1d$^{+/+}$ littermates (**a**, white circle) or B6 (**b**, white circle) mice. (**c**, **d**) CHS response of Jα18$^{-/-}$ mice (black circle) compared with B6 (**c**, white circle) mice or Jα18$^{-/-}$ mice treated on days −1, 0, +1, and +4 with the anti-NK1.1 PK136 mAb (**d**, white circles). (**e**, **f**) CHS in B6 mice either untreated (white circle) or injected with PK136 (black circle) on (**e**) days −3, −2, and −1 before sensitization or on (**f**) days +4, +5, and +6 after sensitization. Results are expressed as the mean ± SD ear swelling at different times after challenge, and are representative of two independent experiments using five to seven mice per group. *P<0.05, **P<0.01, Mann–Whitney U-test.

IFN-γ-producing cytolytic effectors (Kehren *et al.*, 1999). After skin challenge with the hapten, these effectors are recruited into skin and initiate inflammation by inducing keratinocyte apoptosis (Akiba *et al.*, 2002). The intensity and resolution of the CHS response is controlled by regulatory CD4$^+$ T cells (Tregs; Bour *et al.*, 1995), notably CD4$^+$CD25$^+$Foxp3$^+$ ICOS$^+$ Tregs (Dubois *et al.*, 2003; Vocanson *et al.*, 2010) and hapten-induced Th2-type CD4$^+$ T cells (Xu *et al.*, 1996). Here we show that iNKT cells represent a critical regulator of CHS, which inhibits CD8$^+$ T cell–mediated allergic skin inflammation independently of CD4$^+$ Tregs.

RESULTS

iNKT cells control both the afferent and efferent phases of CHS

We previously documented that the defects of CD4$^+$CD25$^+$ Tregs in major histocompatibility complex class II–deficient (Aβ$^{-/-}$) mice resulted in enhanced CHS response to DNFB (Bour *et al.*, 1995; Dubois *et al.*, 2003). In addition, depletion

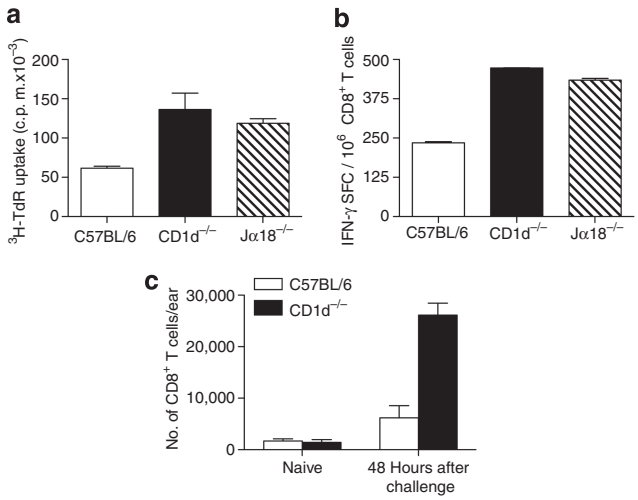


Figure 2. Enhanced priming and skin recruitment of CD8 effectors in CD1d$^{-/-}$ and Jα18$^{-/-}$ mice. (**a**, **b**) Hapten-specific CD8$^+$ T-cell response in lymph nodes (LNs) of day 5 DNFB-sensitized C57BL/6 (white bars), CD1d$^{-/-}$ (black bars), and Jα18$^{-/-}$ (hatched bars) mice. (**a**) Hapten-specific proliferation of CD8$^+$ T cells (mean ± SD of triplicate wells) measured by tritiated thymidine (3H-TdR) uptake. (**b**) Frequency of hapten-specific IFN-γ spot forming cells (SFCs) in draining LNs (dLNs) determined by ELISPOT (mean ± SD SFC/10^6 CD8$^+$ T cells of triplicate wells containing pooled CD8$^+$ T cells from four mice per group). (**a**, **b**) Data are representative of one out of three experiments. (**c**) Number (No.) of CD8$^+$ T cells in the ears of B6 (white bars) and CD1d$^{-/-}$ (black bars) mice at 48 hours after DNFB challenge was determined by FACS analysis. The results show mean ± SD obtained using three mice per group and are representative of one out of two experiments. c.p.m., counts per minute.

of CD4$^+$ T cells in Aβ$^{-/-}$ mice further enhanced the CHS response, pointing out to residual Tregs (Kish *et al.*, 2007). Because CD1d-restricted NKT cells account for most of the residual CD4$^+$ T cells in Aβ$^{-/-}$ mice (Cardell *et al.*, 1995), and mAb depletion of NK1.1$^+$ cells exaggerated their CHS response to DNFB (data not shown), we examined the regulatory potential of NKT cells on the CHS response. CD1d-KO (CD1d$^{-/-}$) mice, deficient in all CD1d-restricted NKT cells (Smiley *et al.*, 1997), exhibited an enhanced CHS response as compared with CD1d$^{+/+}$ littermates or syngeneic wild-type (WT) mice (Figure 1a and b). Importantly, Jα18$^{-/-}$ mice, which specifically lack iNKT cells (Cui *et al.*, 1997), also exhibited an increased CHS (Figure 1c) that was not affected by treatment with the anti-NK1.1 PK136 mAb (Figure 1d), indicating that iNKT cells, but not NK cells, downregulate CHS. Moreover, B6 mice injected with PK136, either before DNFB sensitization to induce NKT cell depletion during both afferent and efferent phases of CHS (Figure 1e) or before DNFB challenge (Figure 1f), similarly displayed an increased ear swelling response, indicating that iNKT cells might negatively control both CD8$^+$ T-cell priming during sensitization and the reactivation of CD8 effectors during elicitation.

Lack of iNKT cells augments the priming of CD8 CHS effectors

Analysis of the hapten-specific CD8$^+$ T-cell response in skin dLNs after DNFB sensitization showed a similar enhancement of hapten-specific proliferation (Figure 2a) and frequency of IFN-γ-producing CD8$^+$ T cells (Figure 2b) in CD1d$^{-/-}$ and

Jα18$^{-/-}$ mice, compared with WT mice. FACS analysis revealed a 4-fold increase of CD8^{+} T-cell numbers in inflamed ears of CD1d$^{-/-}$ mice compared with B6 mice at 48 hours after challenge (Figure 2c). iNKT cells were virtually undetectable up to day 4 after challenge, using mCD1d-α-Galcer tetramers (data not shown). These data show that iNKT cells downregulate the priming of hapten-specific CD8^{+} T cells, resulting in lower numbers of CD8^{+} T-cell effectors recruited into the challenged skin.

iNKT cell transfer normalizes CD8^{+} T cell–mediated CHS response in Jα18$^{-/-}$ mice

To further confirm the regulatory function of iNKT cells on CHS, adoptive transfer experiments were carried out. Intravenous injection of purified liver NKT cells into Jα18$^{-/-}$ mice, 1 day before either hapten sensitization (Figure 3a) or hapten challenge (Figure 3b), similarly decreased the frequency of hapten-specific IFN-γ-producing T cells in dLNs. Moreover, transfer of total liver mononuclear cells, containing 25% α-Galcer tetramer^{+} iNKT cells, into Jα18$^{-/-}$ mice before challenge restored a normal CHS response (Figure 3c), whereas transfer of liver mononuclear cells from naive Jα18$^{-/-}$ mice (e.g., liver cells devoid of iNKT cells) did not (data not shown). These data indicate that the adoptive transfer of iNKT cells can normalize CHS in Jα18$^{-/-}$ mice by controlling both the priming and the reactivation of hapten-specific CD8^{+} effectors.

iNKT cells control CHS independently of CD4^{+}FoxP3^{+} Tregs

To analyze the interplay between iNKT cells and classical CD4^{+}CD25^{+}FoxP3^{+} Tregs for the control of CHS, we examined the impact of NKT cell deficiency on the number, activation state, and functions of Tregs. B6, CD1d$^{-/-}$, and Jα18$^{-/-}$ mice displayed comparable frequencies and numbers of CD8^{+}, CD4^{+}, and Foxp3^{+} cells in spleen and LN (Figure 4a and data not shown), as well as similar activation of CD4^{+}CD25^{+}FoxP3^{+} Tregs after DNFB sensitization (Figure 4b), as measured by the expression of ICOS (Vocanson *et al.*, 2010; Gomez de Aguero *et al.*, 2012). Moreover, reactivation of DNFB-specific Tregs, as assessed by their capacity to proliferate *in vivo* in response to DNFB sensitization (Vocanson *et al.*, 2010), was observed in Jα281$^{-/-}$ mice and was not merely modified by adoptively transferred NKT cells (Figure 4c). In addition, CD4^{+}CD25^{+}ICOS^{+} Tregs were recruited in similar numbers and kinetics as in NKT-sufficient B6 mice (Figure 4d). Standard *in vitro* antigen-specific suppressive assays (Vocanson *et al.*, 2010) further revealed that naive (or DNFB-induced, data not shown) NKT cells did not improve the activity of CD4^{+}CD25^{+} Tregs isolated from either naive or DNFB-sensitized mice (Figure 4e). These data provide compelling evidence that NKT cells do not affect hapten-induced Treg activation, expansion, or functions. Finally, the depletion of CD4^{+} T cells exacerbated the CHS response similarly in CD1d$^{-/-}$, Jα18$^{-/-}$, (Figure 4f) and B6 mice (Bour *et al.*,

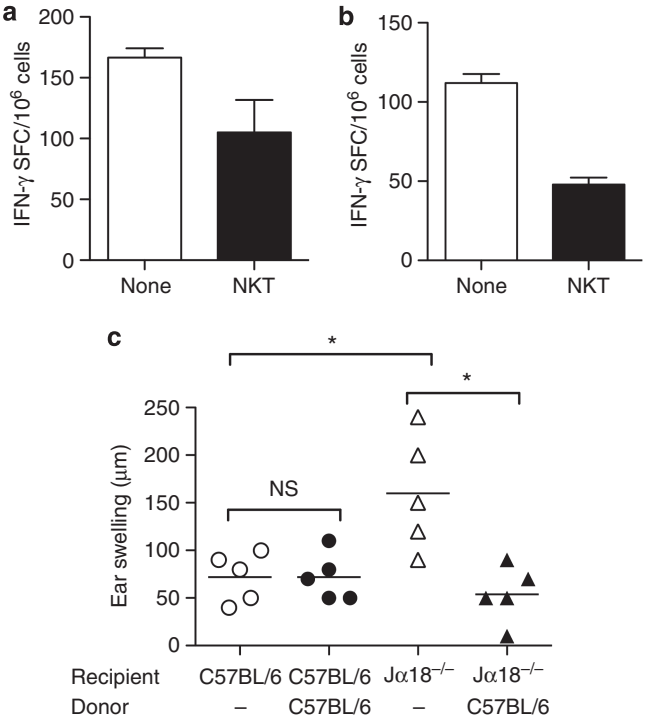


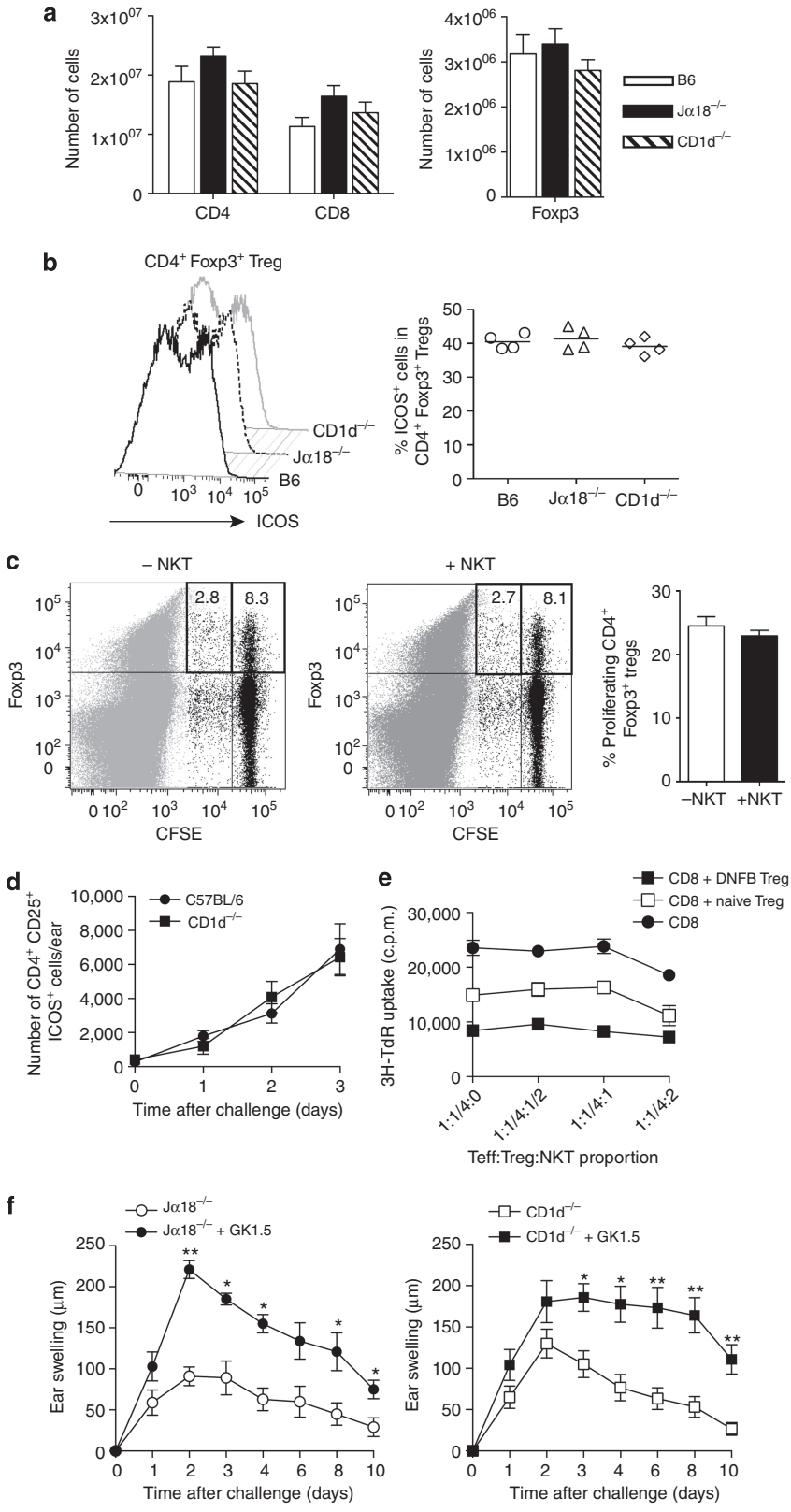
Figure 3. Transfer of invariant natural killer T (iNKT) cells normalizes CD8^{+} T-cell and contact hypersensitivity (CHS) responses in Jα18$^{-/-}$ mice. (**a**, **b**) Frequency of hapten-specific IFN-γ spot forming cells (SFCs) in draining lymph nodes (dLNs) at (**a**) day 5 after sensitization or (**b**) day 2 after challenge in Jα18$^{-/-}$ mice that were either untransferred (white bars) or transferred (black bars) with purified liver naive iNKT cells 1 day before either (**a**) sensitization or (**b**) challenge with DNFB. Data correspond to mean ± SD SFC/10^{6} LN cells of triplicate wells containing pooled LN cells from four mice per group, and are representative of one out of two experiments. (**c**) CHS to DNFB in B6 (circles) and Jα18$^{-/-}$ (triangles) mice either untransferred (white symbols) or transferred 1 day before challenge (black symbols) with 4×10^{6} liver B6 leukocytes. Each symbol represents the ear swelling of individual mouse at 48 hours after challenge and horizontal bars indicate the mean ± SD of 5 mice per group. Data are representative of one out of two experiments. *$P<0.05$, Mann–Whitney test. NS, nonsignificant.

Figure 4. Invariant natural killer T (iNKT) cells control the contact hypersensitivity (CHS) response independently of CD4^{+} regulatory T cells (Tregs). (**a**) Number of splenic CD4^{+}, CD8^{+}, and FoxP3^{+} T cells in B6, Jα18$^{-/-}$, and CD1d$^{-/-}$ mice (mean ± SEM, four mice per group). NS (nonsignificant), Mann–Whitney test. (**b**) CD4^{+}FoxP3^{+}inducible costimulatory molecule (ICOS)$^{+}$Tregs in draining lymph nodes (dLNs) of day 5 sensitized B6, Jα18$^{-/-}$, and CD1d$^{-/-}$ mice. Horizontal bars show mean percentages of four mice (NS, Mann–Whitney test). (**c**) *In vivo* proliferation of carboxyfluorescein diacetate succinimidyl ester (CFSE)$^{+}$CD4^{+}FoxP3^{+} Tregs from day 6 sensitized B6 mice, 3 days after transfer and sensitization in Jα18$^{-/-}$ recipients (black dots) alone or together with naive NKT cells. Gray dots represent endogenous cells. (**d**) Kinetics of post-challenged CD4^{+}CD25^{+}ICOS^{+} Tregs infiltration in the ears of sensitized B6 and CD1d$^{-/-}$ mice (mean ± SEM, three mice per group). (**e**) Suppression of *in vitro* proliferation (measured by uptake of tritiated thymidine [3H-TdR]) of DNFB-specific CD8^{+} T cells (effector T-cell, Teff) by Tregs from DNFB-sensitized mice with or without naive NKT cells. (**f**) CHS in Jα18$^{-/-}$ (circles) and CD1d$^{-/-}$ (squares) mice treated (black symbols) or not (open symbols) with an anti-CD4 mAb. Each symbol represents the mean ± SD of ear swelling values from five to six mice (*$P<0.05$, **$P<0.01$, Mann–Whitney test). c.p.m., counts per minute.

1995). Altogether, these results indicate that NKT cells operate independently of classical $CD4^+FoxP3^+$ Tregs to control CHS responses.

CD1d-deficient DCs exacerbate CHS to DNFB

To examine whether iNKT cell regulation of CHS required interaction with CD1d expressed by DCs, CHS was induced by



subcutaneous immunization with bone marrow–derived DCs (BM-DCs) from WT or CD1d$^{-/-}$ mice. Mice immunized with 2,4-dinitrobenzene-sulfonic acid (DNBS)-pulsed BM-DCs developed a CHS response upon ear challenge with DNFB (Figure 5), confirming our previous report (Krasteva *et al.*, 1998). Interestingly, similarly exacerbated CHS responses were observed in B6 mice immunized with DNBS-pulsed CD1d$^{-/-}$ BM-DCs from CD1d$^{-/-}$ mice and in CD1d$^{-/-}$ mice immunized with DNBS-pulsed BM-DCs from either B6 or CD1d$^{-/-}$ mice. Thus, iNKT cells required CD1d-expressing DCs during sensitization to regulate the CHS response.

IFN-γ from CD8$^+$ T-cell effectors triggers CD1d-dependent secretion of IL-4 and IL-13 by iNKT cells

Because NKT cells can produce Th2-type cytokines, which modulate CHS responses (Xu *et al.*, 1996), we investigated whether hapten-presenting DCs could trigger the production of IL-4 and IL-13 by iNKT cells. Neither IL-4 nor IL-13 could be detected in the supernatant of iNKT cells cocultured with hapten-pulsed BM-DCs alone (Figure 6a). However, addition of CD8$^+$ T cells from DNFB-sensitized mice induced the production of IL-4 and IL-13, but not IFN-γ (data not shown). Production of both cytokines was strongly reduced when cocultures were performed (1) with CD1d-deficient BM-DCs, (2) in the presence of anti-CD1d-neutralizing antibodies (Figure 6a), or (3) with IL-4/IL-13-deficient iNKT cells (Figure 6b), demonstrating that NKT cells were the source of IL-4 and IL-13 and required CD1d expression on DCs to secrete these cytokines. Interestingly, the production of both cytokines by iNKT cells was blocked by the addition of an anti-IFN-γ mAb, and was undetectable in cocultures using CD8 T cells from naive mice, even in the presence of exogenous IFN-γ (Figure 6c). Thus, these data demonstrate that NK-T cells produce IL-4 and IL-13 in response to stimulation through CD1d on DCs, provided that IFN-γ is secreted by specific CD8$^+$ effector T cells.

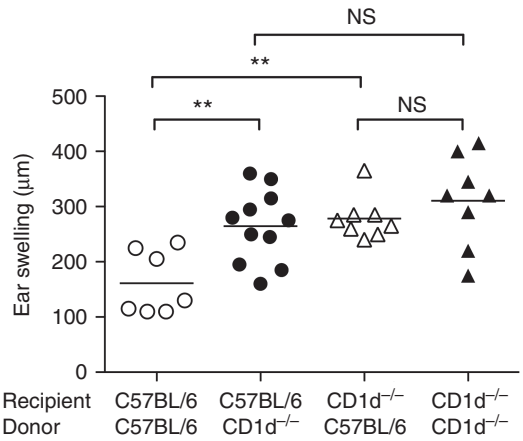


Figure 5. CD1d-deficient dendritic cells (DCs) generate exacerbated contact hypersensitivity (CHS) response to DNFB. CHS response in B6 (circles) and CD1d$^{-/-}$ (triangles) mice immunized with hapten-pulsed bone marrow–derived dendritic cells (BM-DCs) from B6 (white symbols) or CD1d$^{-/-}$ (black symbols) mice and DNFB challenged 5 days later. Each symbol corresponds to the ear swelling of individual mouse at 48 hours after challenge and horizontal bars represent the mean ± SD of 7–13 mice per group. $^{**}P<0.01$, Mann–Whitney test. Data are representative of two independent experiments. NS, nonsignificant.

Enhanced CHS to DNFB in IL-4/IL-13 double-deficient mice

On the basis of the above observations and the fact that iNKT cells can rapidly produce large amounts of IL-4 and IL-13 upon activation *in vivo* (Akbari *et al.*, 2003), we next asked whether these cytokines contributed to the regulation of CHS. Mice double deficient for IL-4 and IL-13 (IL-4$^{-/-}$/IL-13$^{-/-}$) exhibited a significantly increased CHS response to DNFB (Figure 6d). Alternatively, IL-4-deficient mice exhibited a CHS response similar to that of WT B6 mice (data not shown), in agreement with a previous report (Nagai *et al.*, 2000). These data indicated that IL-13, either alone or in synergy with IL-4, can downregulate CHS to DNFB.

DISCUSSION

This study demonstrates that iNKT cells are important actors in the control of CD8$^+$ T-cell-mediated allergic contact dermatitis. We showed that defects in iNKT cells in B6 mice, induced by genetic disruption of either the CD1d molecule or the invariant Vα14Jα18 TCR or by the depletion of NK1.1$^+$ cells, exacerbated CHS to DNFB because of the increased priming of hapten-specific IFN-γ-producing CD8$^+$ T cells in LNs and their enhanced recruitment in the challenged skin. Moreover, reconstitution of Jα18$^{-/-}$ mice with liver iNKT cells before either sensitization or challenge decreased IFN-γ-producing CD8$^+$ T cells in skin dLNs and normalized the CHS response. This demonstrates that iNKT cells control both afferent and efferent phases of DNFB-specific CHS.

Our data corroborate recent studies reporting that iNKT cells inhibit CD8$^+$ T-cell responses in mice in the context of skin graft (Mattarollo *et al.*, 2011) and in virally induced type 1 diabetes (Diana *et al.*, 2011). However, they apparently contrast with previous studies from the group of Askenase, showing a critical inductive role of iNKT cells in CHS. Indeed, in BALB/c mice, sensitization with Picryl chloride (Campos *et al.*, 2003) or DNFB (Askenase *et al.*, 2011) rapidly activates hepatic iNKT cells to release IL-4, which is instrumental for B1 B-cell production of hapten-specific IgM, required for the elicitation of CHS. A similar role of NKT cells has also been proposed in B6 mice sensitized with Oxazolone (Nieuwenhuis *et al.*, 2005). Thus, it is most likely that NKT cells exert a dual function during CHS, as they allow induction of hapten-specific IgM early after sensitization and later on negatively control hapten-specific CD8$^+$ T-cell effectors. The relative importance of these two opposite functions in CHS may depend on the mouse strain, the nature and doses of hapten, and sensitization protocols. It should indeed be emphasized that the inductive role of NKT cells was documented with a quite unusual protocol of CHS involving repeated sensitizations at multiple sites with high doses of hapten, whereas this study was performed with a single conventional dose of hapten on abdominal skin. In addition, it is noteworthy that CHS to DNFB is strongly impaired in BALB/c mice deficient in either IL-4, B cells, or iNKT cells (Askenase *et al.*, 2011), whereas it normally develops in B6 mice deficient in either B cells (μMT) (Seidel-Guyenot *et al.*, 2004, and data not shown) or IL-4 (Nagai *et al.*, 2000, and

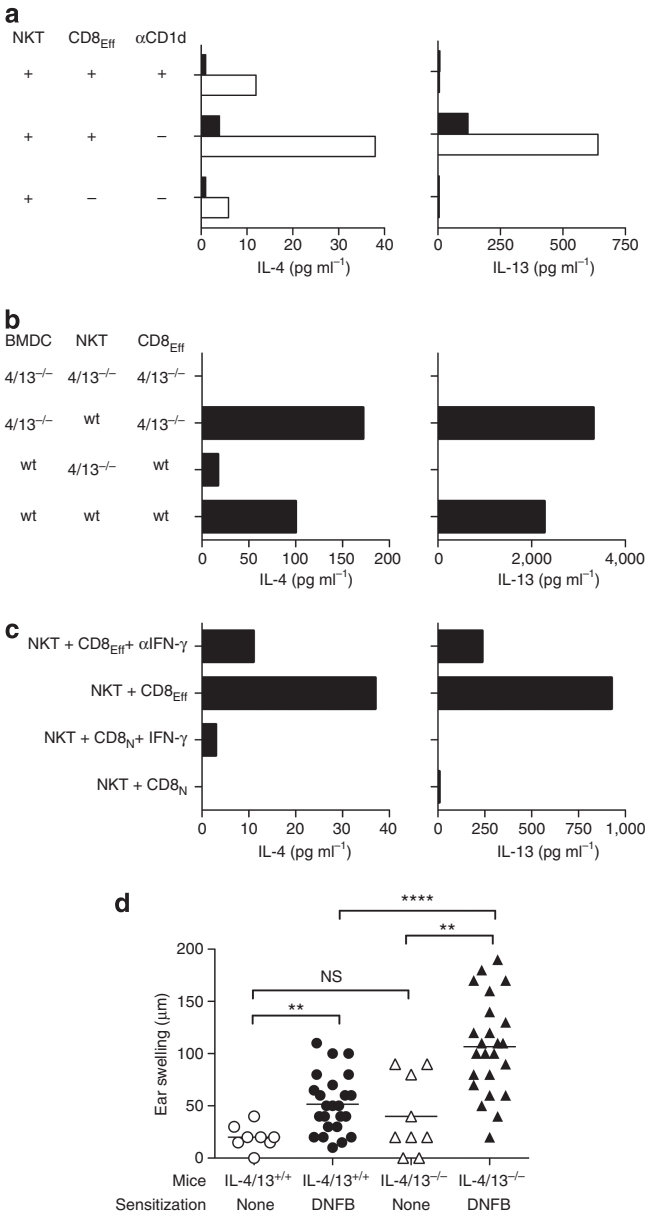


Figure 6. Contact hypersensitivity (CHS) downregulation by invariant natural killer T (iNKT) cell–derived cytokines induced by IFN-γ from CD8$^+$ effectors. (**a**–**c**) IL-4 and IL-13 production by liver iNKT cells was tested in cocultures with (**a**) 2,4-dinitrobenzene-sulfonic acid (DNBS)-pulsed bone marrow–derived dendritic cells (BM-DCs) from B6 (black bars) or CD1d$^{-/-}$ (white bars) mice with or without CD8$^+$ effector T cells (CD8$_{eff}$) and anti-CD1d mAb; (**b**) DNBS-pulsed BM-DCs and CD8$_{eff}$ from either B6 or IL-4/IL-13$^{-/-}$ (4/13$^{-/-}$) mice; and (**c**) hapten-pulsed BM-DCs with naive (CD8$_N$) with or without exogenous IFN-γ or effector (CD8$_{eff}$) CD8$^+$ T cells; wt, wild type. Cytokine released in 72-hour supernatant (pool of triplicate wells) are representative of one out of three experiments. (**d**) Ear swelling in IL-4/IL-13$^{-/-}$ (IL4/13$^{-/-}$; triangles) and IL-4/IL-13$^{+/+}$ (IL4/13$^{+/+}$; circles) mice at 24 hours after DNFB challenge in sensitized (white symbols) or naive (black symbols) mice. Data of individual mice are shown. Horizontal bars indicate mean ± SD of values pooled from three experiments. $^{**}P<0.01$, $^{****}P<0.0001$, Mann–Whitney *U*-test. NS, nonsignificant.

data not shown). This indicates that NKT cells have a prominent inductive role in CHS to DNFB in BALB/c but not B6 mice. Whether NKT cells also display a regulatory role in BALB/c mice and contribute to the control of the effector phase of CHS remains to be determined.

The fact that iNKT cells regulate both the afferent and efferent phases of CHS suggests that they may act both in LN and in skin. However, we observed that, using CD1d-α-GalCer tetramers, iNKT cells were virtually undetectable in the skin before or at any time point after the hapten challenge, a finding in agreement with another study investigating the presence of invariant Vα14Jα18 TCR transcripts (Nieuwenhuis *et al.*, 2005). This suggests that iNKT cells may exert their regulatory function mainly in skin dLNs, during the hapten presentation by the DCs. The fact that enhanced skin inflammation was observed after immunization with hapten-pulsed DCs from CD1d$^{-/-}$ mice as compared with DCs from WT mice, indeed supports that iNKT cells need to interact with DCs to exert their regulatory effects. Along these lines, iNKT cells are activated in LN by contact with CD1d$^+$ epidermal Langerhans cells and mediate UV-induced immune suppression of CHS to DNFB (Fukunaga *et al.*, 2010). The nature of the CD1d ligand expressed on hapten-bearing DCs and responsible for iNKT cell activation remains unknown. These glycolipids could be derived from commensals or be generated endogenously in response to the tissue stress caused by the hapten (Godfrey *et al.*, 2004). In this respect, iNKT stimulatory lipids rapidly accumulate in the liver following hapten sensitization (Dey *et al.*, 2011), although their nature and capacity to activate NKT cells in lymphoid organs remains elusive. Interestingly, CD1d-dependent iNKT cell production of IL-4 and IL-13 requires the presence of hapten-specific CD8$^+$ T-cell effectors and of IFN-γ released by these cells. Such implication of an effector T-cell cytokine in boosting the regulatory pathways is reminiscent of the ability of TNF-α secreted by effector T cells to activate suppressive Foxp3$^+$ Treg cells controlling diabetes (Grinberg-Bleyer *et al.*, 2010). As IFN-γ is promptly released by CD8$^+$ T cells during priming as well as elicitation of CHS (Vocanson *et al.*, 2010), the activation of iNKT cells may take place during cognate DC/CD8 T-cell interactions during the afferent and/or efferent phases of CHS. That IL-13 contributes to the downregulation of CHS is supported by the enhanced skin inflammation in IL-4/IL-13 double-deficient mice (this study), but not IL-4-deficient (Nagai *et al.*, 2000) (and C. Desvignes and Dominique Kaiserlian, unpublished data) mice. However, whether iNKT cells perform their regulatory function through IL-13, as reported earlier for suppression of antitumor immunity (Terabe *et al.*, 2000), remains to be formally demonstrated.

The iNKT-mediated regulation of CHS could involve several mechanisms. The iNKT cells might limit the differentiation of T cells into effectors through cell contacts (Novak *et al.*, 2005) or alter their recruitment to the skin through cytokine production. Indeed, IL-4 and IL-13 can inhibit CD8$^+$ T-cell chemotaxis to CCL5 (Tan *et al.*, 1995), a critical chemokine for CHS (Canavese *et al.*, 2010). Alternatively, iNKT cells might dampen the CD8 T-cell response indirectly by licensing LN DCs for tolerogenic properties (Naumov *et al.*, 2001; Diana *et al.*, 2011; Mattarollo *et al.*, 2011). Finally, iNKT cells may act through generation of Foxp3$^+$ Tregs, which limit the

priming of hapten-specific $CD8^+$ cytotoxic T lymphocyte in LNs (Dubois *et al.*, 2003; Vocanson *et al.*, 2010) and extravasation of CD8 effectors into the skin (Ring *et al.*, 2006). However, our observations (1) that iNKT cells do not affect the proliferation, activation, and functions of $Foxp3^+$ Tregs in response to skin sensitization, and that (2) depletion of either NKT cells in $CD4^+$ T cell–deficient $A\beta^{-/-}$ mice or $CD4^+$ T cells in NKT cell–deficient mice resulted in both cases in exaggerated CHS responses demonstrates that $CD4^+$ Tregs and iNKT cells have nonredundant functions and both control the CHS response. Finally, it would be interesting to determine whether iNKT-mediated suppression of CHS is triggered by type-II NKT cells, which were previously shown to promote anergy and suppressive functions of iNKT in various inflammatory settings (Ambrosino *et al.*, 2007; Halder *et al.*, 2007).

Our data highlight the potential of iNKT cells to regulate even established $CD8^+$ T-cell responses involved in allergic contact dermatitis. As new iNKT cell antigens are starting to emerge, it will be very important to assess their therapeutic potential.

MATERIALS AND METHODS

Mice

Female C57Bl/6 mice were purchased from Charles River laboratories (L'Arbresle, France). $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice on C57BL/6 background were kindly provided by L Van Kaer (Howard Hughes Medical Institute, Nashville, TN) and M Taniguchi (Chiba University, Chiba, Japan), respectively, and were bred as homozygotes. IL-4/IL-$13^{-/-}$ mice and control IL-4/IL-$13^{+/+}$ mice on a C57Bl/6 background were obtained from A McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK) and bred separately as homozygotes. All experiments were conducted at the Plateau de Biologie Expérimentale de la Souris (PBES, SFR Biosciences Gerland-Lyon Sud UMS344/US8, France) under specific pathogen-free conditions with mice between 2 and 4 months of age. They were conducted with the approval of and in accordance with the guidelines for animal experiments of the local ethics committee (CECCAPP Lyon, France).

Antibodies and flow cytometry

The following antibodies were used for flow cytometry analysis: anti-NK1.1-PE (PK136), anti-CD8α-PE-Cy7, anti-biotin (53-6.7), anti-CD5-RPE-Cy5.5 (53-7.3), anti-CD4-FITC (RM4-4), anti-CD45-FITC (30F11), and anti-ICOS-biot (7E.17G9) (all from BD Biosciences, Le Pont de claix, France). Anti-TCRαβ-FITC (H57-597) and CFSE were purchased from Invitrogen (Cergy-Pontoise, France), and anti-FoxP3-PE (FJK-16s) from eBiosciences (Clinisciences, Montrouge, France). Allophycocyanin- mCD1d-α-GalCer tetramers were provided by the NIH tetramer facility (Atlanta, GA). FACS analyses were performed using a FACSCanto or LSR-II flow cytometer (BD Biosciences) and the data analyzed using FlowJo software (Tree Star, Ashland, OR).

Contact hypersensitivity

For most experiments, CHS to DNFB (Sigma-Aldrich, St Quentin Fallavier, France) was induced and measured as previously described (Dubois *et al.*, 2003). Alternatively, mice were sensitized by subcutaneous injection of 0.5×10^6 BM-DCs, which were pulsed with DNBS (Sigma-Aldrich) (Krasteva *et al.*, 1998). Ear swelling in unsensitized but ear-challenged mice was usually $<20\,\mu m$.

NKT and $CD4^+$ T-cell depletion

NKT and $CD4^+$ T-cell depletion were carried out, respectively, by multiple intraperitoneal injections of 200 µl of the anti-NK1.1 (clone PK136, 1/3 dilution of ascites fluid) or anti-CD4 (clone GK1.5, 100 µg per mouse (BioXCell, West Lebanon, NH))-specific mAbs before and/or after DNFB sensitization. Protocols routinely yielded 80% depletion of $NK1.1^+$ cells and >98% depletion of $CD4^+$ cells (data not shown).

FACS sorting and adoptive transfer experiments

Liver leukocytes were prepared as previously described (Goubier *et al.*, 2008), stained with anti-NK1.1-PE and anti-CD5-RPE-Cy5.5 mAbs, and $NK1.1^+CD5^+$ cells were sorted using a FACSVantage cell sorter (BD Biosciences). 5×10^5 purified cells, which were routinely >97% pure and contained >80% $TCR\alpha\beta^+$ CD1d-αGalCer $tetramer^+$ iNKT cells, were injected intravenously into $J\alpha18^{-/-}$ mice 1 day before either sensitization or challenge. Alternatively, total liver leukocytes ($2–4 \times 10^6$) were transferred intravenously 1 day before the challenge.

Hapten-specific T-cell responses

The capacity of $CD8^+$ T cells, isolated from pooled axillary and inguinal LNs of DNFB-sensitized animals, to proliferate and produce IFN-γ in response to *in vitro* stimulation with DNBS-pulsed antigen-presenting cells was determined as previously described (Dubois *et al.*, 2003). The frequency of hapten-specific IFN-γ-producing cells in unfractionated LN cells or purified LN $CD8^+$ T cells was determined by an ELISPOT assay as previously described (Kehren *et al.*, 1999). For suppression assays, $CD8^+$ T cells (5×10^4) from day 5 DNFB-sensitized animals were cultured with DNBS-pulsed BM-DCs (1×10^4) for 3 days, in the presence of $CD4^+CD25^+$ Tregs (1.25×10^4) purified from the same donors or from naive animals, and graded numbers of FACS-sorted naive liver NKT cells.

Coculture of iNKT cells, $CD8^+$ T cells, and DCs

5×10^4 FACS-sorted liver NKT cells were cocultured with DNBS-pulsed BM-DCs (1×10^4) with or without $CD8^+$ T cells (5×10^4) isolated from skin dLNs of DNFB-sensitized mice. In some experiments, anti-CD1d (1B1, $10\,\mu g\,ml^{-1}$) or anti-IFN-γ (R46A2, $10\,\mu g\,ml^{-1}$) neutralizing mAbs (BD Bioscience), or recombinant IFN-γ ($10\,\mu g\,ml^{-1}$) was added to the cocultures. IL-4 and IL-13 secretions were titrated in 72-hour culture supernatants by ELISA (R&D Systems Europe, Lille, France).

Statistics

A nonparametric Mann–Whitney test was used for the assessment of differences between groups. $P<0.05$ was considered significant; NS, nonsignificant. GraphPad Prism 5.0 software (San Diego, CA) was used to prepare graphs and for statistical analysis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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